



8-2016

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## Recommended Citation

Li, N., Yousefi, M., Nakauka-Ddamba, A., Tobias, J. W., Jensen, S. T., Morrisey, E. E., & Lengner, C. J. (2016). Heterogeneity in Readouts of Canonical Wnt Pathway Activity within Intestinal Crypts. *Developmental Dynamics*, 245 (8), 822-833. <http://dx.doi.org/10.1002/dvdy.24423>

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# Heterogeneity in Readouts of Canonical Wnt Pathway Activity within Intestinal Crypts

## Abstract

**BACKGROUND:** Canonical Wnt pathway signaling is necessary for maintaining the proliferative capacity of mammalian intestinal crypt base columnar stem cells (CBCs). Furthermore, dysregulation of the Wnt pathway is a major contributor to disease, including oncogenic transformation of the intestinal epithelium. Given the critical importance of this pathway, numerous tools have been used as proxy measures for Wnt pathway activity, yet the relationship between Wnt target gene expression and reporter allele activity within individual cells at the crypt base remains unclear.

**RESULTS:** Here, we describe a novel *Axin2-CreERT2-tdTomato* allele that efficiently marks both Wnt<sup>High</sup> CBCs and radioresistant reserve intestinal stem cells. We analyze the molecular and functional identity of *Axin2-CreERT2-tdTomato*-marked cells using single cell gene expression profiling and tissue regeneration assays and find that *Axin2* reporter activity does not necessarily correlate with expression of Wnt target genes and, furthermore, that Wnt target genes themselves vary in their expression patterns at the crypt base.

**CONCLUSIONS:** Wnt target genes and reporter alleles can vary greatly in their cell-type specificity, demonstrating that these proxies cannot be used interchangeably. Furthermore, *Axin2-CreERT2-tdTomato* is a robust marker of both active and reserve intestinal stem cells and is thus useful for understanding the intestinal stem cell compartment.

## Keywords

crypt base columnar stem cells, quiescent stem cells, reserve stem cells, Wnt signaling, Bmi1, Hopx, Lgr5, intestine

## Disciplines

Biology | Business | Cell and Developmental Biology | Genetics and Genomics | Statistics and Probability

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Published in final edited form as:

*Dev Dyn.* 2016 August ; 245(8): 822–833. doi:10.1002/dvdy.24423.

## Heterogeneity in readouts of canonical Wnt pathway activity within intestinal crypts

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### Abstract

**Background**—Canonical Wnt pathway signaling is necessary for maintaining the proliferative capacity of mammalian intestinal crypt base columnar stem cells (CBCs). Further, dysregulation of the Wnt pathway is a major contributor to disease, including oncogenic transformation of the intestinal epithelium. Given the critical importance of this pathway, numerous tools have been utilized as proxy measures for Wnt pathway activity, yet the relationship between Wnt target gene expression and reporter allele activity within individual cells at the crypt base remains unclear.

**Results**—Here we describe a novel *Axin2-CreERT2-tdTomato* allele that efficiently marks both Wnt<sup>High</sup> CBCs and radioresistant reserve intestinal stem cells. We analyze the molecular and functional identity of *Axin2-CreERT2-tdTomato*-marked cells using single cell gene expression profiling and tissue regeneration assays and find that *Axin2* reporter activity does not necessarily correlate with expression of Wnt target genes, and, further, that Wnt target genes themselves vary in their expression patterns at the crypt base.

**Conclusions**—Wnt target genes and reporter alleles can vary greatly in their cell-type specificity, demonstrating that these proxies cannot be used interchangeably. Further, *Axin2-CreERT2-tdTomato* is a robust marker of both active and reserve intestinal stem cells and is thus useful for understanding the intestinal stem cell compartment.

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<sup>7</sup>These authors contributed equally to this work

## Keywords

Crypt base columnar stem cells; quiescent stem cells; reserve stem cells; reserve stem cells; Wnt signaling; *Bmi1*; *Hopx*; *Lgr5*; intestine

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## INTRODUCTION

The intestinal epithelium is a highly dynamic tissue, undergoing complete turnover approximately every four days. Numerous recent studies have identified the intestinal stem cells (ISCs) that drive this process using a variety of genetic proxy reporter alleles. Taken together, these studies lead to a model in which at least two functionally and molecularly distinct stem cells reside at the base of the intestinal crypt, maintaining normal tissue turnover during homeostasis and rapid epithelial regeneration upon injury (Li and Clevers, 2010). This model proposes that the daily proliferative demand of the epithelium is met by an actively cycling crypt base columnar ISC (CBC) driven by high activity of the canonical Wnt pathway and often marked experimentally by expression of an *Lgr5-eGFP-IRES-CreER* reporter allele (Barker et al., 2007). The high activity of the Wnt pathway and rapid proliferation of the CBC stem cells, however, sensitizes them to DNA damage, which causes them to undergo apoptosis (Tao et al., 2015, Yan et al., 2012). In addition to CBCs, a second, largely quiescent (residing in G0) (Li et al., 2016) population of Wnt<sup>Negative</sup> reserve ISCs periodically divides to give rise to new CBCs during homeostasis. In contrast to CBCs, these reserve ISCs are highly radioresistant and can be identified by CreER knockin alleles at the endogenous *Bmi1* and *Hopx* loci, which mark largely overlapping populations (Li et al., 2014, Sangiorgi and Capecchi, 2008, Takeda et al., 2011, Tian et al., 2011, Yan et al., 2012, Li et al., 2016). Reserve ISCs can also be identified by transgenes driven by the *mTert* promoter; however the degree of overlap between the *mTert*-marked populations and the *Hopx-/Bmi1-CreER* marked population has never been directly investigated (Montgomery et al., 2011).

Here we apply single cell analysis techniques to study the population of epithelial cells marked by a novel *Axin2-CreERT2-tdTomato* knockin reporter allele. The *Axin2* gene encodes a protein that interacts with APC in the destruction complex responsible for degradation of  $\beta$ -catenin in the absence of Wnt ligand stimulation of the canonical pathway, and thus acts as a potent negative regulator of Wnt signaling (Kishida et al., 1998). *Axin2* itself is a direct transcriptional target of  $\beta$ -catenin, thus creating a negative feedback loop for canonical Wnt pathway activity (Jho et al., 2002). Because of this, numerous studies in the intestinal epithelium have used *Axin2* expression and *Axin2* reporter mice as a surrogate readout for activity of the canonical Wnt pathway, and thus, by extension, as a proxy for active intestinal stem cells (Lustig et al., 2002, Kim et al., 2007, van Amerongen et al., 2012). It is, however, becoming increasingly clear that various Wnt reporter alleles can behave quite differently from one another [for example, the *Axin2-LacZ* reporter relative to TOPGAL and BATGAL reporters (Al Alam et al., 2011)]. Further, it is also evident that even direct  $\beta$ -catenin target genes can vary in their expression pattern. For example, within the small intestines, large secretory Paneth cells residing at the crypt base intercalated between CBCs require Wnt activity for their maturation and exhibit clear nuclear  $\beta$ -catenin

staining, express the Wnt target gene *Sox9*, but lack expression of other intestinal Wnt target genes such as *Lgr5* and *Ascl2* (van Es et al., 2005, Li et al., 2015, Wang et al., 2015, van der Flier et al., 2009, Blache et al., 2004, Andreu et al., 2005, Andreu et al., 2008). Thus, the identities of the precise populations of cells with active canonical Wnt signaling and how these relate to those marked by the Wnt/ $\beta$ -catenin target gene *Axin2* at the base of intestinal crypts remains somewhat ambiguous. Here, we attempt to clarify the identity of these cells using functional and single-cell molecular approaches.

## RESULTS

In order to study the molecular identity and functional role of intestinal epithelial cells expressing *Axin2*, we utilized a novel *Axin2-CreERT2-tdTomato* knockin reporter allele in which a *CreERT2-2A-tdTomato* polycistronic cassette is knocked into the translational start site of the endogenous *Axin2* locus (Figure 1A)(Choi et al., 2013). Gross analysis of tdTomato expression along the entirety of the adult small intestine revealed a craniocaudal gradient of reporter activity with highest tdTomato intensity in the duodenum (proximal small intestine), decreasing throughout the jejunum, and lowest in the ileum (distal small intestine) (Figure 1B). This observation is consistent with previous observations of a craniocaudal gradient of Wnt activity made using distinct detection methods (Davies et al., 2008). Closer inspection of individual crypt-villus units revealed that tdTomato reporter activity is restricted to the crypt, with highest activity at the crypt base that gradually decreases through the transit-amplifying zone and is extinguished at the crypt-villus junction (Figure 1C). Interestingly, *Axin2* reporter activity was absent from large crypt base cells whose position and appearance was consistent with Paneth cells, despite these cells having strong nuclear  $\beta$ -catenin immunoreactivity and their known dependence on canonical Wnt signaling for their differentiation (Andreu et al., 2005, Andreu et al., 2008, van Es et al., 2005), (Figure 1D, arrows), in contrast to nuclear  $\beta$ -catenin<sup>Neg</sup> cells higher in the transit-amplifying compartment (Figure 1D, arrowheads). To confirm the absence of *Axin2* reporter activity in Paneth cells, we costained for tdTomato and the Paneth cell phenotypic protein Lysozyme, which further supports their mutual exclusivity (Figure 1E).

*Axin2* is frequently used as a surrogate for activity of the canonical Wnt pathway, which in the intestinal crypt is highly active in CBC stem cells. An *Lgr5-eGFP-IRES-CreER* knockin reporter allele is commonly used to mark CBC stem cells; however this allele is known for its mosaic expression pattern, being active only in roughly 30% of small intestinal crypts (Barker et al., 2007, Li et al., 2014). We intercrossed the *Axin2-CreERT2-tdTomato* and *Lgr5-eGFP-IRES-CreER* alleles and confirmed the existence of tdTomato/eGFP double positive cells by flow cytometry (Figure 1F). To better understand the expression of *Axin2-CreERT2-tdTomato* within the *Lgr5*<sup>+</sup> compartment, we calculated the frequency of tdTomato<sup>+</sup> cells within *Lgr5-eGFP<sup>High</sup>* CBCs and within the total *Lgr5-eGFP<sup>+</sup>* compartment containing both CBCs and *Lgr5-eGFP<sup>Med-Low</sup>* cells that primarily represent CBC daughter cells that are entering the transit-amplifying (TA) zone (van der Flier et al., 2009, Munoz et al., 2012). We found *Lgr5-eGFP<sup>High</sup>* CBCs to be uniformly tdTomato<sup>+</sup>, and the bulk *Lgr5-eGFP<sup>+</sup>* population (including *Lgr5-eGFP<sup>Med-Low</sup>* cells in the TA compartment) to be nearly uniformly tdTomato<sup>+</sup> (Figure 2A, B). The converse calculation (% of *Axin2-tdTomato<sup>+</sup>* cells

that are *Lgr5-eGFP<sup>+</sup>*) cannot, however, be performed due to the mosaicism of the *Lgr5-eGFP-IRES-CreER* allele.

To functionally evaluate the stem cell activity and dynamics of *Axin2-CreERT2-tdTomato*-marked cells, we intercrossed these mice with a *ROSA26-Lox-Stop-Lox-tdTomato* reporter strain, such that after Tamoxifen (Tam) treatment, both cells expressing *Axin2* and their progeny are irreversibly labeled with *tdTomato*. We found that within 24 hours of Tam treatment, the progeny of *Axin2-CreERT2-tdTomato* cells began migrating up towards the crypt-villus junction. Between 48–72 hours, *Axin2-CreERT2-tdTomato* progeny had uniformly reached the tip of the villi, and complete crypt-villus units remained fully labeled thereafter (Figure 2C). These findings confirm that *Axin2-CreERT2-tdTomato* uniformly labels CBC stem cells and, given that this allele is not mosaic (Figure 1B), it may provide more utility relative to the *Lgr5-eGFP-IRES-CreER* allele when homogenous and efficient target gene deletion in the crypt is desired.

Given the craniocaudal gradient in *tdTomato* expression we observed along the alimentary canal in Figure 1B, we examined Cre-mediated recombination efficiency from the proximal small intestine to the distal colon one week after a single Tamoxifen injection in *Axin2-CreERT2-tdTomato::ROSA26-Lox-Stop-Lox-tdTomato* mice and found that the epithelium exhibited uniform recombination along the entire axis (Figure 3). This indicates that despite the decreased expression of *Axin2-CreERT2-tdTomato* caudally, there is still sufficient CreERT2 produced to drive highly efficient recombination.

In order to more fully understand the heterogeneity within the *Axin2-CreERT2-tdTomato*-marked population and the types of ISCs expressing this allele, we performed single-cell gene expression analysis on a random population of FACS-purified *tdTomato<sup>+</sup>* epithelial cells, examining the expression of genes encoding Wnt and Notch pathway targets and components, ISC signature genes, proliferation/metabolic-related genes, markers of differentiated cells, and housekeeping genes, using two distinct primer sets per gene (Li et al., 2014) (Figure 4A, B, and Supplemental Table 1). Analysis of gene expression patterns in single *tdTomato<sup>+</sup>* cells arbitrarily split into *tdTomato<sup>High</sup>* and *tdTomato<sup>Low</sup>* groups reassuringly reveals that *tdTomato<sup>High</sup>* cells exhibit higher expression levels of Wnt target genes such as *Myc*, *Ascl2*, *Lgr5*, and *Axin2* itself relative to the *tdTomato<sup>Low</sup>* group (Figure 4B). We next compared the identity of *Axin2-CreERT2-tdTomato*-marked cells to those of active CBCs (marked by *Lgr5-eGFP*) and reserve ISCs (marked either by *Bmi1-* or *Hopx-CreER* knockin alleles)(Li et al., 2014). The identity of these different cell populations was visualized using Principal Component Analysis, where the position of each individual cell is a representation of its gene expression profile (Figure 5A, B). This analysis revealed the *Axin2-CreERT2-tdTomato* population to be highly heterogeneous relative to these two distinct stem cell populations. As expected, the *Axin2-CreERT2-tdTomato* population largely overlapped with the CBC population, based on histological and flow cytometric overlap with *Lgr5-eGFP* (Figure 5A). Interestingly, there was also significant overlap between *Axin2-CreERT2-tdTomato*-marked cells and the *Bmi1-* or *Hopx-CreER*-marked reserve stem cell populations [which themselves are largely overlapping, (Li et al., 2014)] (Figure 5A, B). These data indicate that, in addition to labeling *Lgr5-eGFP* active CBCs, the *Axin2-CreERT2-tdTomato* allele also labels the reserve stem cell compartment.

Recent studies have demonstrated that high Wnt activity and basal crypt positioning sensitizes CBCs to DNA damage such as that which occurs after exposure to high dose ionizing radiation (Tao et al., 2015, Yan et al., 2012). In response to CBC loss, Wnt<sup>Negative</sup> reserve stem cells are induced to proliferate to replenish the depleted CBC compartment (Tian et al., 2011, Yan et al., 2012, Li et al., 2014, Li et al., 2016). In addition, genetic ablation of CBCs can also open the niche allowing for their downstream progeny to revert to the CBC state (Tetteh et al., 2016). We therefore sought to test whether *Axin2-CreERT2-tdTomato* marks functional reserve/facultative stem cells that contribute to regeneration. We initiated lineage tracing prior to exposure to ionizing radiation and then asked whether the regenerating epithelium was derived from *Axin2-CreERT2-tdTomato*-marked cells. To do this, we initially administered a single dose of Tamoxifen to *Axin2-CreERT2-tdTomato::ROSA26-Lox-Stop-Lox-tdTomato*, then allowed for a 48-hour chase period prior to exposure to 12 Gy whole-body gamma irradiation ( $\gamma$ -IR), followed by a 72-hour recovery period during which highly proliferative regenerative crypt foci form. The 48-hour chase period prior to  $\gamma$ -IR should minimize any post- $\gamma$ -IR *de novo* reporter allele recombination due to the persistence of Tamoxifen in the bloodstream given its 12–18 hour half-life (Robinson et al., 1991). Analysis of regenerative crypt foci 72 hours after 12 Gy  $\gamma$ -IR revealed that regenerative foci were tdTomato<sup>+</sup>, indicating that these foci either actively and uniformly express *Axin2-CreERT2-tdTomato*, or were lineage-traced from these cells (Figure 5C). To distinguish these possibilities, we performed a similar experiment, analyzing the epithelium 4 days after irradiation instead, a timepoint where fully regenerated crypt-villus units can be observed. This revealed that the regenerated epithelium, including differentiated villi, was uniformly labeled with the tdTomato reporter, indicating that radioresistant *Axin2-CreERT2-tdTomato*-marked cells are capable of driving regeneration after radiation injury (Figure 5D). Thus, these findings demonstrate that the *Axin2-CreERT2-tdTomato* efficiently labels not only CBCs, but also radioresistant reserve/facultative stem cells responsible for regeneration in the face of CBC loss.

While active CBCs are well-known to exhibit high activity of the canonical Wnt pathway and thus expected to be marked by the *Axin2-CreERT2-tdTomato* allele, reserve ISCs are known to have little to no canonical Wnt activity in their resting state, but give rise to Wnt<sup>High</sup> CBCs upon cell cycle entry (Tian et al., 2011, Takeda et al., 2011, Li et al., 2014). Thus, our observations indicating that reserve ISCs are also marked by the *Axin2* reporter was unexpected. Conversely, Paneth cells that exhibit nuclear  $\beta$ -catenin and expression of some Wnt target genes such as Sox9 were unexpectedly not marked by the *Axin2* reporter. To understand the relationship between Wnt target gene expression and *Axin2-CreERT2-tdTomato* reporter activity in these various cell populations within intestinal crypts, we examined single cell gene expression profiles in CBCs, reserve ISCs, and Paneth cells. Paneth cells were purified based on cell-surface CD24 and c-Kit immunoreactivity, using a c-Kit antibody that has previously been vetted for Paneth cell specificity (Rothenberg et al., 2012). As expected, Lgr5<sup>+</sup> CBCs exhibited very high expression of *Axin2* (Figure 6). Interestingly, cells within the other populations marked by *Axin2-CreERT2-tdTomato* including reserve ISCs and the bulk *Axin2-tdTomato*<sup>+</sup> population (which includes TA cells) exhibit variable *Axin2* mRNA expression, possibly reflecting cells either just entering a Wnt<sup>On</sup> state (such as the reserve ISCs upstream of CBCs) or just exiting the Wnt<sup>On</sup> state

(such as the TA cells downstream of CBCs) (Figure 6). Conversely, Paneth cells that are *Axin2-tdTomato*<sup>Neg</sup> and express high levels of the phenotypic genes *Lyz2* and *Wnt3a* also contain abundant *Axin2* and *Sox9* mRNA along with nuclear  $\beta$ -catenin, but exhibit few cells containing appreciable *Ascl2*, *Lgr5*, and *Ccnd1* transcripts (genes associated with canonical Wnt pathway activity in CBCs) (Figure 6).

Finally, we utilized unsupervised hierarchical clustering of all single cell expression profiles from Paneth cell, reserve ISCs, CBCs, and *Axin2-CreERT2-tdTomato*<sup>+</sup> cells to get a global view of the relationship between and the heterogeneity within these intestinal crypt populations (Figure 7A). This clustering first confirms that CBCs and reserve ISCs are molecularly distinct populations with little overlap. Next, it reveals the heterogeneity with the *Axin2-CreERT2-tdTomato*-marked population, showing overlap of this population with both the active CBCs and reserve ISCs, and exclusivity from Paneth cells. It is important to note here that none of these alleles mark truly homogenous populations: rare reserve ISCs can be found clustering with CBCs, rare *Lgr5-eGFP*<sup>High</sup> CBCs can be found clustering with Paneth cells, etc. Ultimately, we conclude that there is no prototypical Wnt pathway target gene or proxy reporter that faithfully captures *all* cells with an active canonical Wnt pathway in intestinal crypts, and *only* cells with an active canonical Wnt pathway (Figure 7B).

## DISCUSSION

Activity of the canonical Wnt pathway is critical for proper function of not only the intestinal epithelium, but all epithelial tissues. In the intestinal crypts, high Wnt activity drives the proliferation of the crypt base columnar stem cells, and, reassuringly, our analysis confirms that these cells are positive for all readouts of Wnt pathway activity which we examined, including high expression of target genes such as *Axin2*, *Ascl2*, *Ccnd1*, *Lgr5*, and *Sox9*, as well as high levels of *Axin2-CreERT2-tdTomato* reporter expression and nuclear  $\beta$ -catenin immunoreactivity. Thus, all of these metrics can be considered markers of the CBC state. What is becoming increasingly clear, however, is that most of these markers are not *specific* for CBCs. Thus, understanding the expression patterns of surrogate markers of Wnt pathway activity at single cell resolution within the intestinal crypt is crucial for proper interpretation of phenotypes that are associated with perturbations in the expression of these markers or use of Cre recombinase alleles driven by Wnt target genes (including *Axin2-CreERT2-tdTomato*).

In the current study we employ a novel *Axin2-CreERT2-tdTomato* reporter allele, along with reporter alleles that mark CBCs (*Lgr5-eGFP-IRES-CreER*) and reserve ISCs (*Bmi1-* and *Hopx-CreER*), as well as with markers of Paneth cells (*c-Kit/CD24*<sup>+</sup>) to generate a detailed picture of the expression patterns of commonly used Wnt proxy reporters. The *Axin2-CreERT2-tdTomato* provides additional functional capability over prior *Axin2* alleles containing only CreERT2 or LacZ which do not allow for FACS-purification of live cells with an active *Axin2* locus (Lustig et al., 2002, van Amerongen et al., 2012). Interestingly, we find that *Axin2-CreERT2-tdTomato* marks a large, heterogeneous population of cells at the crypt base, including cells that do not exhibit other hallmarks of Wnt pathway activity such as reserve ISCs and transit-amplifying cells. The vast majority of reserve ISCs lack expression of canonical Wnt targets such as *Lgr5*, *Ascl2*, *CyclinD1*, and *Sox9*, and these



cells tend to reside at and above the Wnt<sup>+</sup> zone at the crypt base. Despite this, reserve ISC populations contain a significant fraction of cells expressing *Axin2*, and are marked by the *Axin2-CreERT2-tdTomato* allele. The transit-amplifying compartment also resides above the Wnt<sup>+</sup> zone and lacks nuclear  $\beta$ -catenin immunoreactivity traditionally associated with canonical Wnt pathway activation. Cells in this region, however, are positive for *Axin2-CreERT2-tdTomato*, although tdTomato immunoreactivity progressively decreases moving upward in the TA zone, and tdTomato<sup>Low</sup> almost uniformly lack *Lgr5* mRNA, suggesting that the *Axin2-CreERT2-tdTomato* present in the TA zone may be due to perdurance of the protein rather than active *Axin2* transcription. This phenomenon is consistent with the observed eGFP perdurance in *Lgr5-eGFP<sup>Low</sup>* cells. Conversely, the Wnt<sup>Negative</sup> reserve ISCs marked by *Bmi1*- or *Hopx-CreER* are known to reside upstream of the Wnt<sup>High</sup> CBC stem cells (Li et al., 2014, Takeda et al., 2011, Tian et al., 2011, Yan et al., 2012). Thus, the tdTomato<sup>Low</sup> state may reflect either the TA cell progeny of CBCs that have very recently inactivated the *Axin2* locus, or the immediate progeny of Wnt<sup>Negative</sup> reserve ISCs just turning on the *Axin2* locus as they enter the CBC state. This would account for the larger variation observed in the *Axin2-tdTomato<sup>Low</sup>* population relative to the *Axin2-tdTomato<sup>High</sup>* population and the partial overlap of the former, but not the latter population with *Hopx-CreER* and *Bmi1-CreER* reserve ISC populations in the PCA analyses.

The most confounding cell type at the crypt base with respect to interpreting activity of the canonical Wnt pathway are the post-mitotic Paneth cells intercalated with the CBCs at the crypt base. These cells are known to produce Wnt ligands to support their neighboring CBCs (as is evidenced by the very high and specific *Wnt3* expression in our single cell analysis of CD24/c-Kit<sup>+</sup> Paneth cells). Paneth cells also exhibit clear nuclear  $\beta$ -catenin immunoreactivity and express high levels of Wnt target genes *Axin2* and *Sox9* along with the *Wnt3* ligand. This suggests that in addition to providing niche signals to CBCs, Paneth cells also engage in cell-autonomous canonical Wnt signaling, and it is well-established that canonical Wnt signaling contributes to Paneth cell differentiation (Andreu et al., 2005, Andreu et al., 2008, van Es et al., 2005). Curiously, Paneth cells do not express *Ascl2* and rarely express *Lgr5*, suggesting either that inhibitory mechanisms exist to prevent expression of these Wnt targets, but not others, or that additional co-factors exist in CBCs to support expression of *Ascl2*, *Lgr5*, and similar targets. Most confounding is the fact that Paneth cells appear to not be marked by the *Axin2-CreERT2-tdTomato* allele despite the other hallmarks of canonical Wnt pathway activity they exhibit, including the presence of high *Axin2* mRNA levels. Similarly, it appears that the widely-used *Axin2-LacZ* allele does not label Paneth cells either, although the diffuse nature of X-gal staining make interpretation of *Axin2-LacZ* expression at the single cell level challenging (Park et al., 2009, Johnson et al., 2013, Lustig et al., 2002). It is entirely unclear why the discordance between *Axin2* mRNA and *Axin2* reporter allele activity exists in Paneth cells.

In summary, the current study highlights the variability and inconsistency between broadly used readouts of canonical Wnt pathway activity in functionally distinct epithelial cell populations at the crypt base. Further, it demonstrates the utility of a highly efficient *Axin2-CreERT2-tdTomato* allele for Cre-mediated recombination of target genes in both active and reserve stem cells of the intestinal epithelium.

## MATERIALS AND METHODS

### Mouse Strains

*Lgr5-EGFP-IRES-CreER* (JAX mice strain 008875), *Bmi1-CreER* (JAX strain 010531), and *R26-CAG-LSL-tdTomato* (JAX mice strain 007914) mice were obtained from the Jackson Laboratory. *Hopx-CreER* (JAX strain 017606) mice were generated at the University of Pennsylvania. Mice were maintained on a C57/BL6N background. *Axin2-CreERT2-tdTomato* mice were generated by insertion of a *CreERT2/tdTomato* fusion cDNA downstream of the first ATG of the mouse *Axin2* gene using homologous recombination in mouse ESCs, described in detail in (Choi et al., 2013). For CreERT2 induction experiments, mice received 1–3 daily intraperitoneal injections of 100  $\mu$ l Tam (10 mg/ml in corn oil; Sigma; T5648). All mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania under protocol 803415 to Dr. Lengner.

### Histology, Immunofluorescence, and Immunocytochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4°C. Subsequently, tissues were washed in PBS and then moved to 70% ethanol before paraffin embedding and sectioning. Hematoxylin and eosin staining was performed in the Morphology Core of the Penn Center for Molecular Studies in Digestive and Liver Diseases according to standard procedures. For immunostaining, antigen-retrieval was performed by heating slides in 10mM citrate buffer (10mM Sodium citrate, 0.05% Tween 20, pH 6) or Tris/EDTA buffer (10 mM Tris base, 1mM EDTA solution, 0.05% Tween 20, pH 9) with a pressure cooker.

The following primary antibodies were used for immunostaining: Living Colors DsRed Polyclonal Antibody (1:200, Clontech, 632496), Anti- $\beta$ -Catenin antibody (1:1000, Sigma, C7207), Anti-E-cadherin (1:200, Invitrogen, 13-1900), and Anti-Lysozyme C Antibody (C-19)(1:200, Santa Cruz, sc-27958). In panel 1E, where tdTomato and Lysozyme are co-stained, we utilize Clontech DsRed Monoclonal (632392) to avoid cross-reactivity with the Lysozyme antibody raised in rabbit. In experiments not shown, direct comparison of Clontech monoclonal (632392) versus polyclonal (632496) antibodies revealed that the monoclonal antibody exhibits some non-specific background staining in the lamina propria (1E). This is not seen with the polyclonal antibody (1B, C), and both of these antibodies give identical staining patterns in the epithelium. Cy2-, Cy3-, and Cy5- conjugated secondary antibodies were obtained from the Jackson Immunoresearch Laboratories. Biotinylated secondary antibodies and DAB substrate kit for immunohistochemistry were purchased from Vector Labs. All photomicrographs depicting *Axin2-tdTomato+* cells are acquired using antibodies to detect tdTomato.

### Flow Cytometry and Single-Cell Sorting

Intestinal epithelial cells were isolated as described previously (Sato et al., 2009), with modifications. Small intestine (jejunum) was dissected out of mice of the appropriate genotype, opened longitudinally, and washed twice with cold PBS. The tissue was incubated in 5mM EDTA/HBSS for 10 min at 4°C and then the villi were gently scraped off using glass cover slip. The intestine was minced and placed into fresh 5mM EDTA/HBSS and incubated for 30 minutes on ice. After transfer of tissue into fresh EDTA/HBSS buffer, the

tissue fragments were vigorously suspended by pipetting. The supernatant was centrifuged at 1500 rpm for 5 minutes to collect crypts. To generate a single cell suspension, crypts were incubated with Accutase (BD Biosciences, San Jose, CA) at 37°C for 10min. Flow cytometry analysis was performed with BD LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). DAPI negative cells were selected, then gated for single cell based on Forward-scatter height versus forward- scatter width (FSC-H vs FSC-W) and side-scatter height vs. side-scatter width (SSC-H vs. SSC-W) profiles. Epithelial cells were selected based on E-cadherin cell surface staining. Single-cell sorting experiments was performed with BD FACSAriaII cell sorter, each single cell was sorted into a different well of a 96-well PCR plate, using the FACSAriaII flow cytometer software package (FACSDiva) with single cell precision mode. Paneth cell isolation was done based on CD24 (eBioscience, 12-0242081)) and c-Kit (eBioscience, 25-1171-81) double staining. The size of the nozzle for all sorting is 100 µm (20 psi). Axin-tdTomato<sup>+</sup> and Lgr5<sup>+</sup> stem cells were quantified by flow cytometry on cells isolated from *Axin2CreERT2-tdTomato::Lgr5-eGFP-IRES-CreER* mice. 1µg/ml DAPI was used to exclude dead cells from the quantification of ISCs.

### Fluidigm Single-Cell Gene Expression

All single cells were sorted 18 hours after activation of the R26R-Lox-Stop-Lox-tdTomato reporter allele using a single dose of Tamoxifen (1mg in 100ul of corn oil).

The two-step single-cell gene-expression protocol (advanced development protocol 33) from Fluidigm was adopted for this study. Briefly, 5 µl of RT Mix Solution which includes 1.2 µl 5× VILO Reaction Mix (Life Technologies; 11754-250), 0.3 µl SUPERase-In (Life Technologies; AM2696), and 0.25 µl 10% NP40 (Thermo Scientific; 28324) was dispensed into each well of 96-well plate. Single cells were sorted into the well directly. The plate was vortexed and immediately frozen on dry ice. For room temperature cycling, the plate was thawed on ice and RNA denatured by incubating at 65°C for 90 s and then chilled on ice for 5 min. Each well was supplemented with 1 µl mixture of 10× SuperScript Enzyme Mix (Life Technologies; 11754-250) and T4 Gene 32 Protein (New England BioLabs; PN M0300S). mRNA was reverse transcribed into cDNA following the thermal cycling conditions below: 25°C, 5 min/50°C, 30 min/55°C, 25 min/60°C, 5 min/70°C, and 10 min. Resulting cDNA was preamplified with 50 nM primer mix for 23 PCR cycles (96°C for 5 s and 60°C for 4 min) and then treated with ExoI for 30 min to remove unincorporated primers. The final product was diluted 1:3 with Tris-EDTA (TE) buffer. For each chip sample inlet, 2.25 µl diluted cDNA, 2.5 µl 2× Sso Fast EvaGreen supermix with low ROX, and 0.25 µl of Fluidigm sample loading agent were added. Individual gene-specific DELTAgene assays were diluted at 1:10 ratios with TE buffer. Two and a half microliters of each primer was then mixed with 2.5 µl assay loading agent inserted into chip “assay” inlets. Chip loading and PCR was performed according to the manufacturer’s protocol. The data were analyzed by Fluidigm Gene Expression Analysis Package. Raw data for the single cell expression analysis of the active and reserve stem cells can be found in (Li et al., 2014)

Genes analyzed include: *Areg, Asc12, Atoh1, Axin2, Bmi1, Bmpr1a, Ccnd1, Cdkn1a, Cdx1, Chga, Cubn, Dll4, Dvl2, Efnb1, Epas1, Ephb2, Ereg, Fut2, Gapdh, Gsk3b, Gusb, H6pd,*

*Hes1, Hes5, Hif1a, Hopx, Jag1, Lgr5, Lrig1, Lyz2, Msi1, Msi2, Myb, Myc, Notch1, Numb, Olfm4, Pcna, Ppargc1b, Rhoa, Saa2, Sirt3, Sox9, Tat, Tcf4, Tert, Wnt3, and Wnt6.*

### Violin Plots

Violin plots were generated as follows. For each cell, we have a measure of the cycle time for 96 primer sets (48 genes with duplicate primer sets). A cycle value of 30 was imputed for any cycle values that did not amplify by 30 cycles (i.e., no signal). For each gene, violin plots were constructed using the statistical software R to compare the distribution of cycle times for that gene between the conditions. For PCA analysis, Fluidigm Ct values were averaged for each gene (across the two primer sets per gene) in each sample. Principal Components Analysis (PCA, using Partek Genomics Suite v6.6, Partek, Inc. St. Louis, MO) was used to visualize the global variation across the samples. Samples were colored to represent their condition. Statistical significance of differences between the mean expression values between populations was calculated using an independent sample t-test.

### Hierarchical Clustering

Figure 7 shows the hierarchical clustering of 468 cells from 5 different populations. For all possible pairs of these 468 cells, we calculate the Pearson correlation of the cycle values (across all 96 primer sets) between each pair of cells in all cell populations. We then create a hierarchical clustering tree that groups cells together based on the correlation of their expression. Cells (or groups of cells) that are highly correlated (correlation near 1) have a distance near 0 from each other and are clustered together near the bottom of the tree. Cells (or groups of cells) that are less correlated (correlation less than 0.5) have a distance near 0.5 and are clustered together only at the top of the tree.

The clustering in Figure 7 is visualized as a tree where each cell is a colored line that indicates the proxy reporter used to isolate that particular cell. The Y-axis gives the distance between the cells at different levels of the tree. Distances near 0 imply correlations of near 1 between those groups of cells; distances near 0.6 imply correlations of only 0.4 between those groups of cells. The R package 'hclust' was used (with the average linkage setting) to create the hierarchical clustering.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

We would like to thank Sarah Rauer in the UPenn Department of Biomedical Sciences for assistance with graphical illustration. C.J.L. is funded by R01 CA168654 from the National Cancer Institute and R01 DK106309 from the National Institute of Diabetes and Digestive and Kidney Diseases. M.Y. is supported by a Howard Hughes Medical Institute International Student Research Fellowship. This work was supported in part by the NIH/NIDDK Center for Molecular Studies in Digestive and Liver Diseases (P30DK050306) and its core facilities (molecular pathology and imaging, molecular biology/gene expression, cell culture, and mouse), and C.J.L. was supported by the center's pilot and feasibility grant program.

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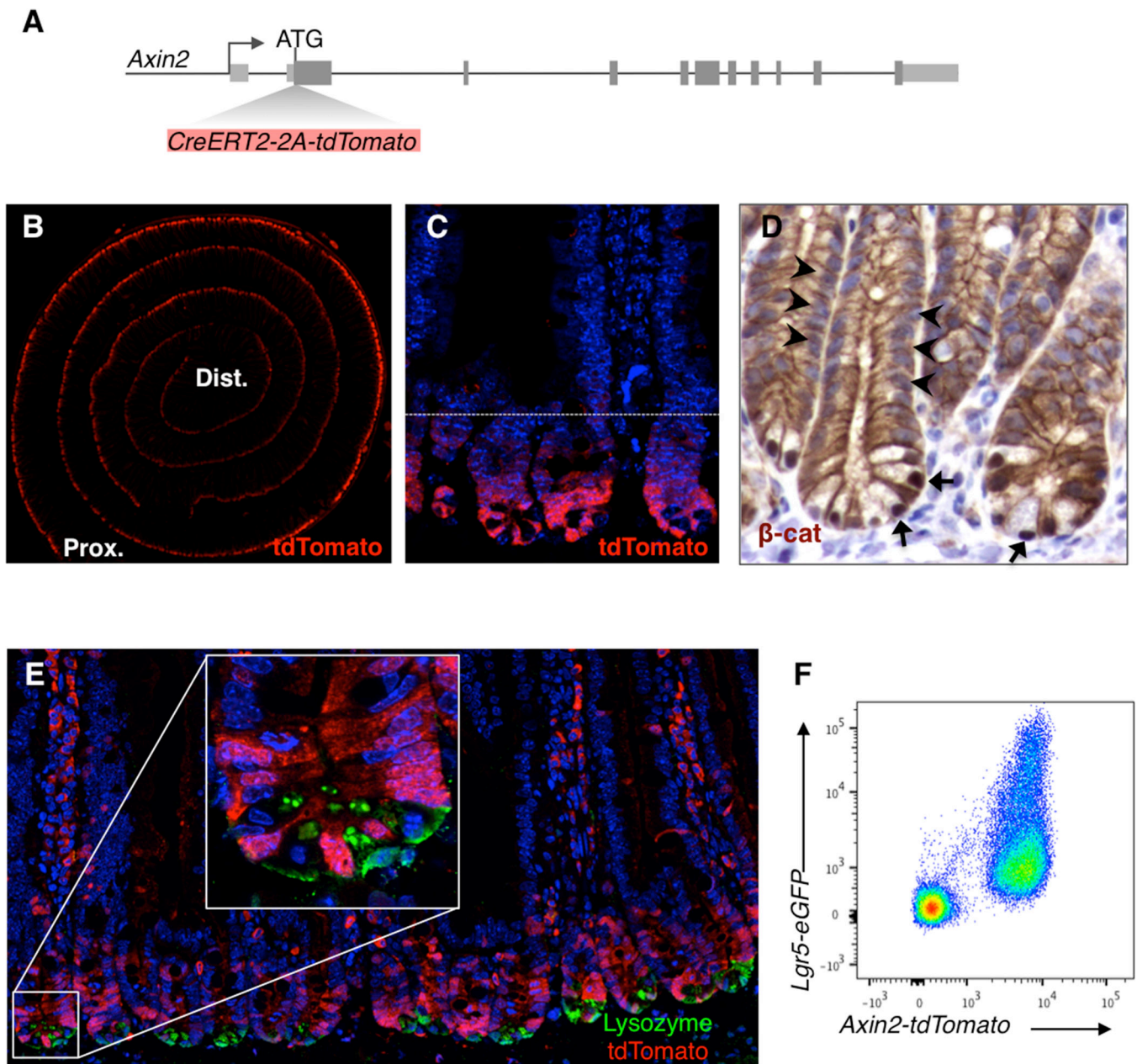
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**Figure 1. Activity of an *Axin2-CreERT2-tdTomato* allele in the small intestinal epithelium**

**A.** Schematic of reporter allele with a cDNA encoding a CreERT2-tdTomato fusion protein inserted into the translational start site of the endogenous *Axin2* locus. **B.** Swiss roll of the entire small intestine illustrating a cranio-caudal gradient of tdTomato reporter fluorescence. Prox.= proximal small intestine (Duodenum), Dist.= distal small intestine (Ileum). **C.** tdTomato fluorescence is restricted to the base of intestinal crypts of the jejunum. The dashed line indicates the crypt-villus junction. **D.** Immunohistochemical staining for  $\beta$ -Catenin in jejunal crypts. Arrows indicate nuclear  $\beta$ -Catenin in Paneth cells at the crypt base. Arrowheads indicate cells lacking nuclear  $\beta$ -Catenin immunoreactivity. **E.** Costaining for tdTomato (Red) and Lysozyme (Green) indicates a lack of *Axin2-CreERT2-tdTomato*



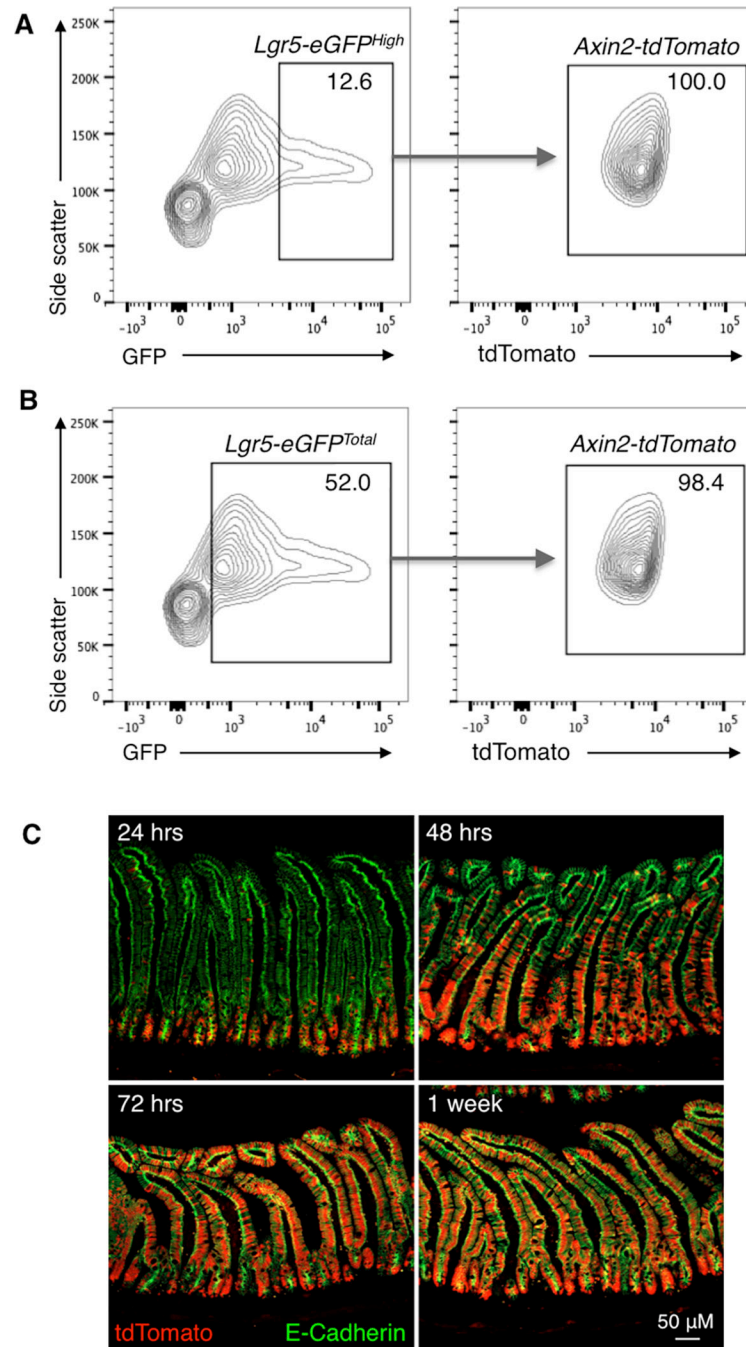
reporter activity in Lysozyme<sup>+</sup> Paneth cells within the jejunum. **F.** Flow cytometric analysis of jejunal crypt cells coexpressing eGFP (y-axis) and tdTomato (x-axis) in *Lgr5-eGFP-IRES-CreER::Axin2-CreERT2-tdTomato* compound mice. 100,000 crypt cells are analyzed.

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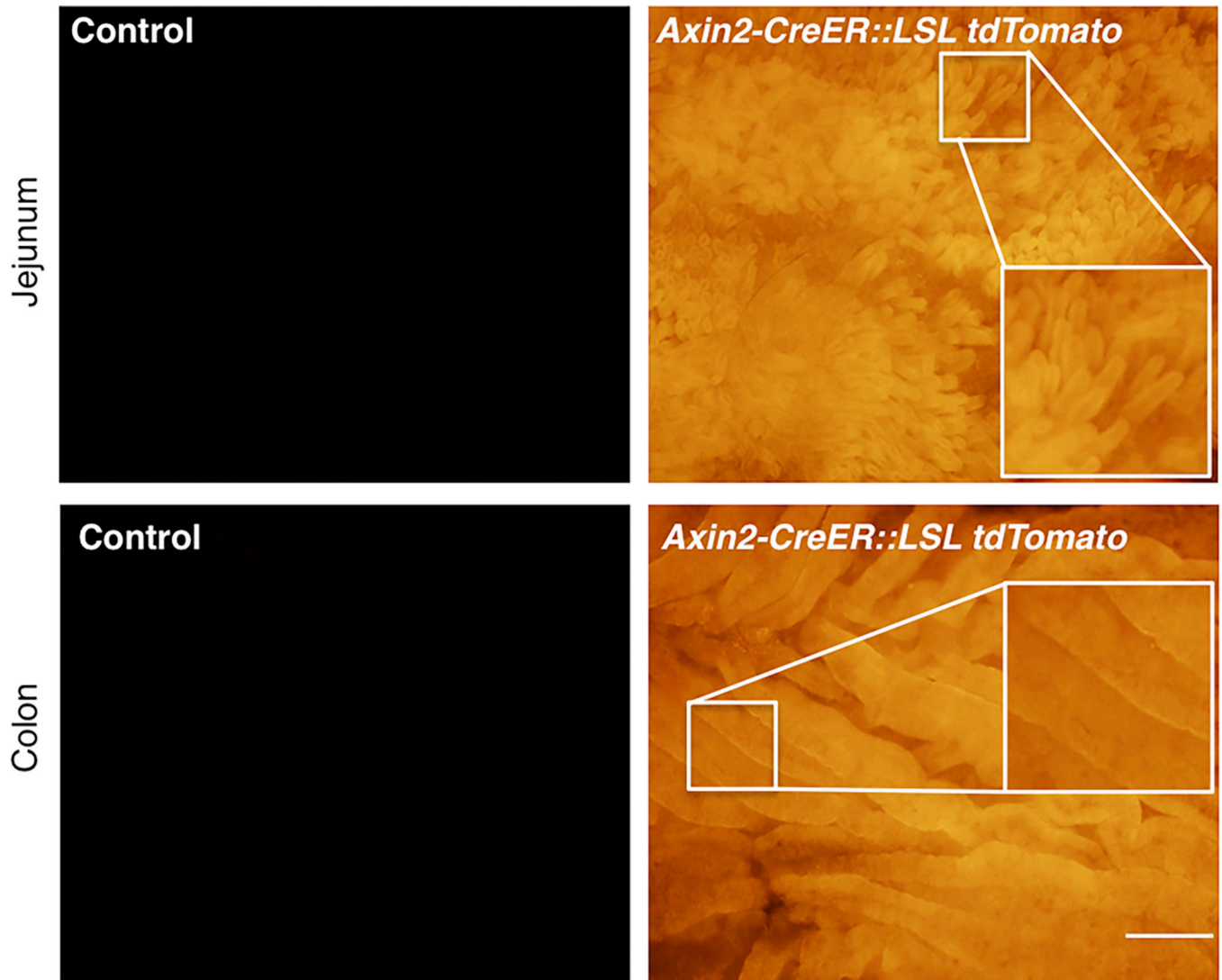
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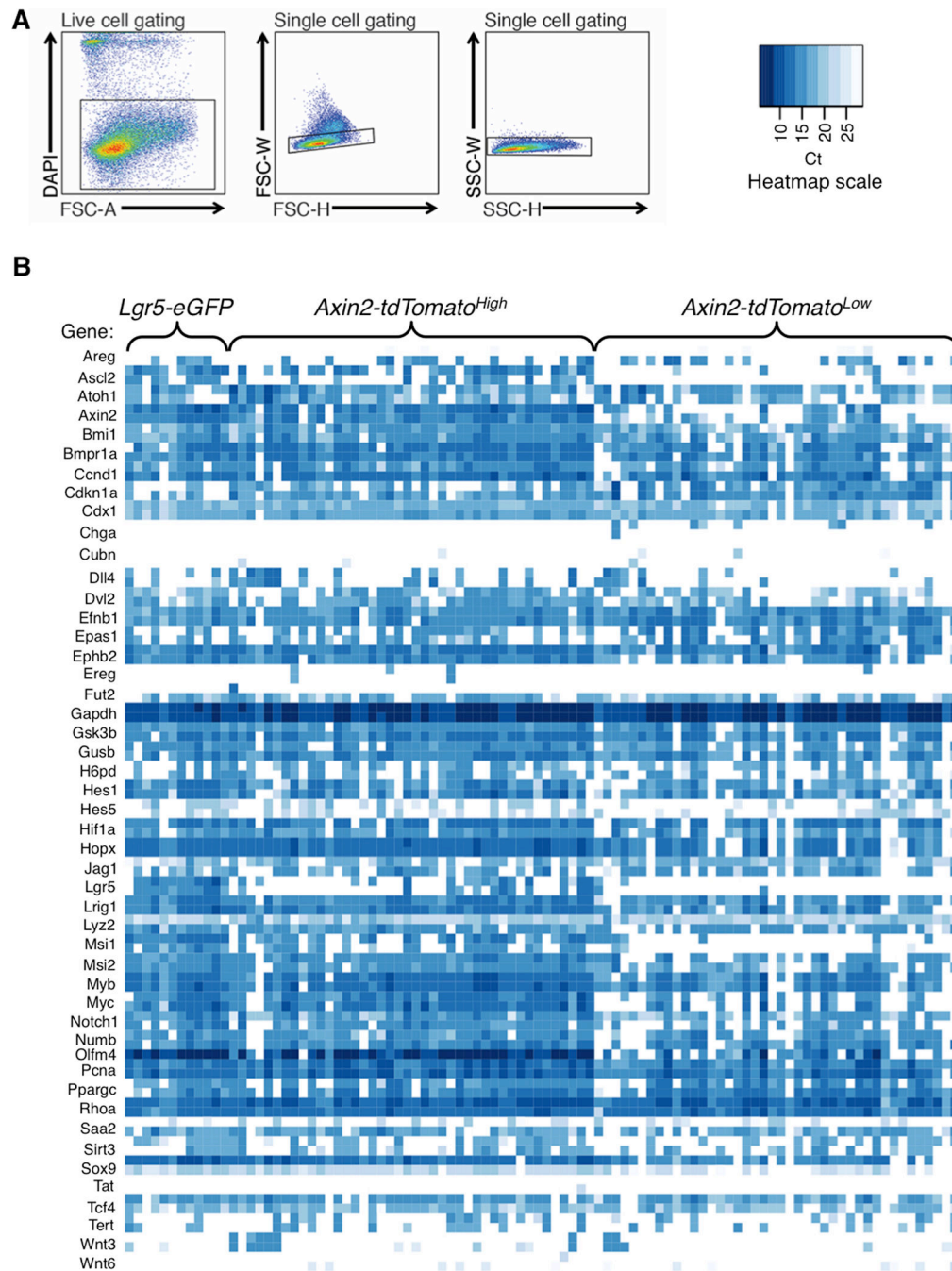


**Figure 2. *Axin2-CreERT2-tdTomato* marks *Lgr5-eGFP*<sup>+</sup> cells including CBCs**  
**A–B.** Flow cytometric analysis of *Axin2-CreERT2-tdTomato* in populations gated on *Lgr5-eGFP*<sup>High</sup> CBC stem cells (**A**), and the bulk *Lgr5-eGFP*<sup>+</sup> population isolated from crypts of the jejunum (**B**). A total of 100,000 cells are scored in **A** and **B**. **C.** Lineage tracing in the jejunum from *Axin2*-expressing cells in *Axin2-CreERT2-tdTomato::R26Lox-Stop-Lox-tdTomato* mice after a single dose of Tamoxifen at the indicated timepoints. E-Cadherin is stained in green to outline epithelial cells. All data derived from the jejunum of adult mice.



**Figure 3. Tamoxifen-induced CreER recombination from the *Axin2-CreERT2-tdTomato* allele is highly efficient along the alimentary tract**

*Axin2-CreERT2-tdTomato::R26Lox-Stop-Lox-tdTomato* or control mice were given a single Tamoxifen injection and the entire alimentary tract was visualized using whole-mount direct fluorescence. One week after CreER activation, the entirety of the alimentary tract, including the proximal jejunum (top panel) distal colon (lower panel) were efficiently labeled with the activated *R26Lox-Stop-Lox-tdTomato* reporter. Scale bar= 1mm.



#### Figure 4. Single cell sorting and gene expression profiling

**A.** Gating strategy for isolation of single cells for Fluidigm Biomark single cell gene expression profiling. FSC: forward scatter. SSC: side scatter. **B.** Heatmap of single cell gene expression on the Fluidigm Biomark device. Columns represent single cells, and rows represent individual primer sets, with two primer sets used to detect each of the indicated genes (with the exception of *Olfm4*, represented by a single primer set). Total *Axin2-CreERT2-tdTomato*<sup>+</sup> cells were sorted and analyzed, then retroactively annotated as being in the *tdTomato*<sup>Low</sup> versus *tdTomato*<sup>High</sup> grouping (top and bottom 50% of total *tdTomato*<sup>+</sup>

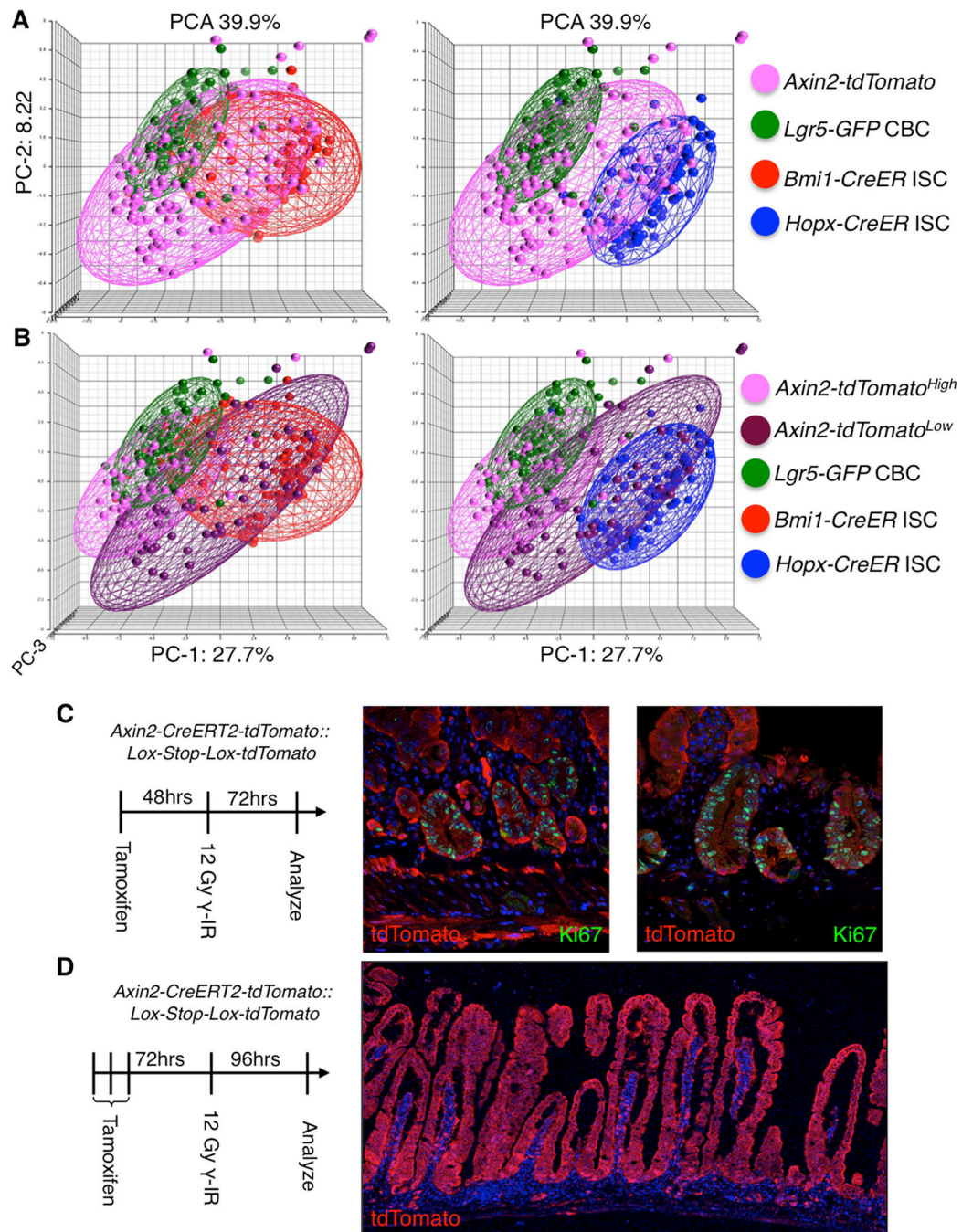
cells). A small group of *Lgr5-eGFP<sup>+</sup>* CBCs are included in the heatmap for reference. Color intensity of the heatmap directly correlates with decrease Ct values. All data derived from the jejunum of adult mice.

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**Figure 5. *Axin2-CreERT2-tdTomato* marks radioresistant reserve intestinal stem cells**

**A–B.** Principal Component Analysis (PCA) of single cells [84 *Axin2-tdTomato* (divided into 42 high, 42 low), 93 *Bmi1-CreER*, 91 *Hopx-CreER*, 105 *Lgr5-eGFP-CreER*] purified based on their expression of the indicated reporter alleles. The position of each cell (sphere) on the PCA plot is a representation of its identity based on the expression of 48 genes (see Methods and Materials for full gene list). For all groups analyzed, the *R26-Lox-stop-Lox-tdTomato* reporter is activated 18 hours before cell isolation. We have previously demonstrated that the *eGFP* and *CreER-Lox-stop-Lox-tdTomato* reporters in *Lgr5-eGFP-CreER::R26-Lox-stop-*

*Lox-tdTomato* mice mark overlapping populations (Li et al., 2014). **C.** *Axin2-CreERT2-tdTomato::R26Lox-Stop-Lox-tdTomato* mice were exposed to 12 Gy of  $\gamma$ -IR 2 days after Tamoxifen administration. tdTomato/Ki67 expression was analyzed in the regenerated epithelium 3 days after irradiation. **D.** *Axin2-CreERT2-tdTomato::R26Lox-Stop-Lox-tdTomato* mice were exposed to 12 Gy of  $\gamma$ -IR 3 days after Tamoxifen administration. tdTomato expression was analyzed in the regenerated epithelium 4 days after irradiation. All data derived from the jejunum of adult mice.

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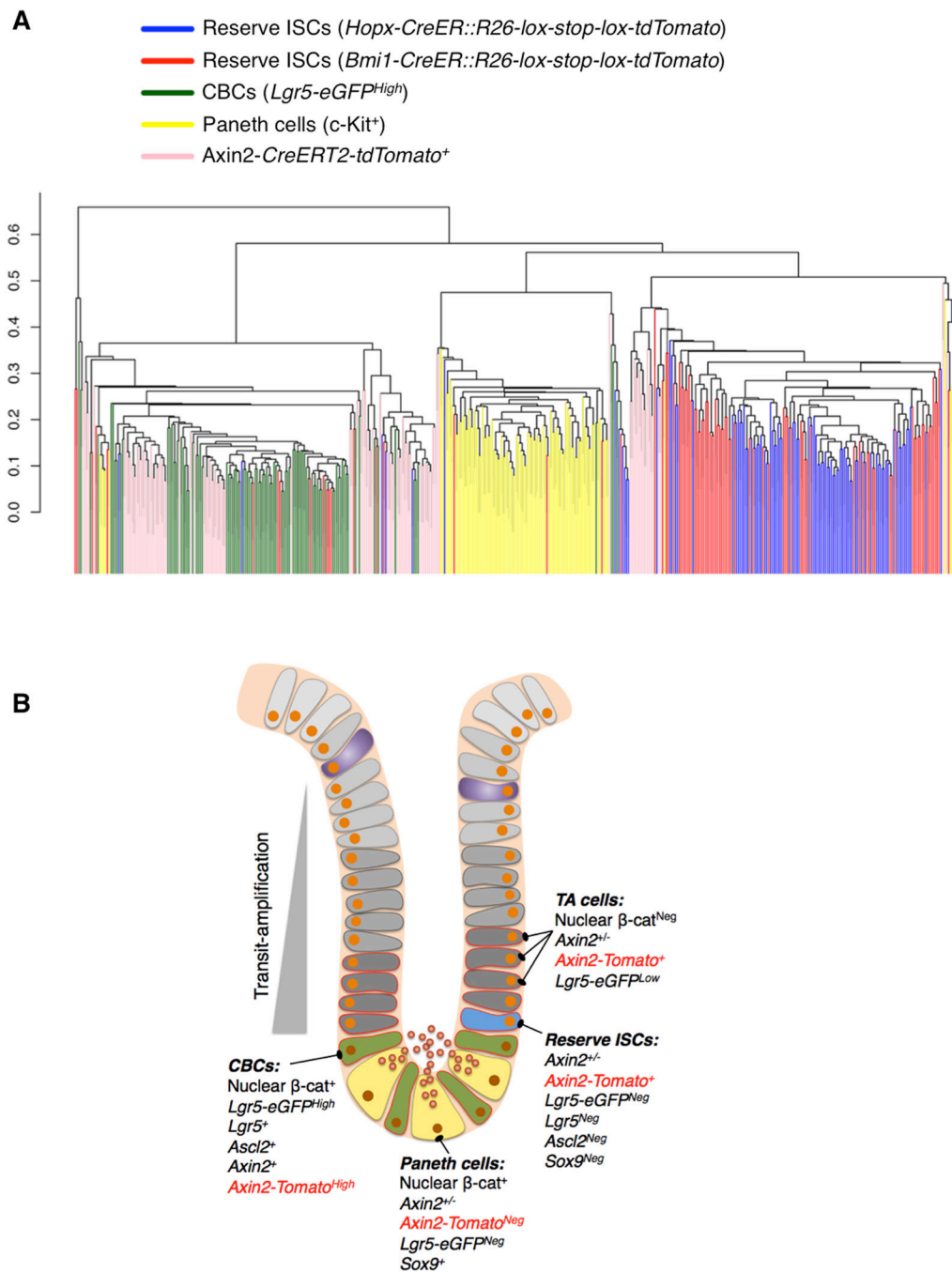
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**Figure 7. Relationship between epithelial populations at the small intestinal crypt base**  
**A.** Unsupervised hierarchical clustering of single cells from indicated populations based on gene expression profiles. **B.** Unified model of canonical Wnt pathway proxy marker expression in cells of the small intestinal crypt. All data derived from the jejunum of adult mice.